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PURIFICATION OF INSULIN-LIKE PEPTIDES FROM INSECT HAEMOLYMPH AND ROYAL JELLY*

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Abstract—Insulin-like peptides from the haemolymph of the tobacco hornworm, *Manduca sexta* L. and from royal jelly of the honey bee, *Apis mellifera* L , were purified by acid extraction, ion-exchange chromatography, gel filtration and affinity chromatography Quantities detected were 500 and 25 pg porcine insulin equivalents per gram of lyophilized material for haemolymph and royal jelly, respectively *M. sexta* insulin was similar to vertebrate insulin in solubility, chromatographic, immunological and biological properties. Amino acid compositions were comparable except for serine, leucine and phenylalanine The *A mellifera* peptide had similar properties, but was not isolated in large enough quantity to determine its amino acid composition These results demonstrated that insect insulin and vertebrate insulin are structurally similar.

Key Word Index Insulin-like peptide, haemolymph, royal jelly, immunoassay, *Manduca sexta, Apis mellifera*, affinity chromatography

INTRODUCTION

NEUROENDOCRINE tissues and haemolymph from the tobacco hornworm, *Manduca sexta* L. and royal jelly from the honey bee, *Apis mellifera* L. contain insulin-like polypeptides (TAGER *et al.*, 1976; KRAMER *et al.*, 1977; KRAMER *et al.*, 1980). The hornworm peptide modulates trehalose levels when injected into the larval form of the same species while the honeybee factor also promotes glucose oxidation in vertebrate adipose tissue (DIXIT and PATEL, 1964). Recently, evidence that a hypolipaemic peptide from the locust, *Locusta migratoria*, and a lipogenetic peptide from *Drosophila melanogaster* have some molecular features in common with mammalian insulin has been presented (ORCHARD and LOUGHTON, 1980, LE ROITH *et al.*, 1980). We report here the purification of insulin-like peptides from haemolymph of the tobacco hornworm and royal jelly of the honeybee.

MATERIALS AND METHODS

Insects, royal jelly and haemolymph collection

Eggs of *Manduca sexta* were a gift from Dr J. Reinecke (Agricultural Research, U S Department of Agriculture, Fargo, ND , U S A). Larvae were reared at 28°C and 60% r.h during a 16 hr L–8 hr D photoperiod on a standard diet (BELL and JOACHIM, 1976). Royal jelly from *Apis mellifera* was obtained from Dr Nevin Weaver (Department of Biology, University of Massachusetts, Boston) and from Formosa Joy Enterprises Co (Taiwan). Upon receipt, royal jelly was immediately lyophilized and stored under nitrogen at −20°C until used. Approximately 0.4 g of freeze-dried jelly was the equivalent of 1 g of native material

Haemolymph was collected from chilled fifth-instar larvae after anaesthesia by cutting off the abdominal horn at its base It was immediately frozen, lyophilized, and stored at −20°C.

Extraction

Twenty grams of freeze-dried haemolymph (equivalent of about 400 ml fresh haemolymph) was extracted with 100 ml of 3 M acetic acid. The suspension was centrifuged at 3000 *g* and 4°C for 20 min. The supernatant was decanted and saved Another 60 ml of 3 M acetic acid was mixed with the precipitate and the centrifugation step repeated. The two supernatants were combined, frozen and lyophilized to yield about 4.4 g dry weight.

Two hundred grams of freeze-dried royal jelly were solubilized in 1 l. of 1 mM ammonium acetate, pH 7 7 containing 1 mM diisopropylphosphorofluoridate (DFP) to prevent proteolytic degradation. After incubation at 4°C for at least one hour, the sample was partially delipidated by extraction with 360 ml of diethyl ether The biphasic mixture was then centrifuged at 7000 *g* and 4°C for 30 min, after which the upper ether layer and interfacial material were discarded The aqueous phase was acidified with acetic acid to give a final concentration of 3 M. The solution was centrifuged at 4°C and 7000 *g* for 30 min and the supernatant was retained and lyophilized to dryness.

Cation-exchange chromatography

The haemolymph extract was dissolved in 1.5 M acetic acid and applied to a 2 0 cm i.d × 30 cm column of sulphopropyl Sephadex C-25 (Pharmacia, Uppsala) equilibrated in the same buffer Fractions of 2 ml each were eluted at 1.6 ml/min After washing the column with several column volumes of equilibration buffer, adsorbed material was eluted with 0 5 M NaCl and was monitored by absorbance at 280 nm. Adsorbed and nonadsorbed material were pooled separately, dialyzed using Spectrapor tubing (mol wt cut off 2000) against 0.1 M acetic acid, frozen and lyophilized.

Gel filtration

Dried samples (Sephadex C-25 fraction II from haemolymph and the supernatant fraction from royal jelly extract) were dissolved in 20–40 ml of 3 M acetic acid and applied to a column of Bio-Gel P-10 (BioRad, Richmond, CA, 2 5 cm i.d. × 45 cm, haemolymph) or P-30 (2.5 cm

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1 d × 90 cm, royal jelly) previously equilibrated with the same solvent. Fractions were collected (3–5 ml) and their absorbance at 280 nm obtained using a Cary 118C spectrophotometer. Samples were pooled, dried under vacuum to remove solvent, and aliquots were subjected to radioimmunoassay.

Affinity chromatography

Porcine insulin antibody was immobilized on an agarose matrix following the method of AKANUMA *et al* (1970). Four millilitres of goat anti-porcine insulin serum (Research Products, Elk Grove, IL) was mixed with ammonium sulphate to give a solution of 18% salt. Precipitate was collected after centrifugation at 3000 *g* for 5 min, dissolved in water and dialyzed against 0·14 M NaCl. The total amount of immunoglobulin in the dialysate was 28 mg as determined by 280 nm absorbance (SOBER, 1970). A 5·4 g sample of cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) was coupled to the immunoglobulin in 30 ml of 0·1 M $NaHCO_3$, pH 8 containing 0·5% NaCl. After washing twice with coupling buffer, the beads were incubated with 30 ml of 1 M ethanolamine, 0·1 M $NaHCO_3$, pH 8, to react with any unmodified electrophilic sites on the beads. Noncovalently adsorbed material was removed by washing first with 0·1 M sodium acetate, 1 M NaCl, pH 4 buffer and then with 0·1 M sodium borate, 1 M NaCl, pH 8 buffer. The anti-insulin immunoglobulin coupled Sepharose (abbreviated 'AIS') was stored at 4°C in the latter buffer also containing 1% gentamicin sulphate (Schering, Port Reading, NJ) as a preservative. Of the immunoglobulin 97% was coupled to the Sepharose as determined by the change in absorbance at 280 nm. The AIS was divided into three portions, one for characterization and the other two for purification of *Manduca* and *Apis* insulin-like peptides.

Binding specificity and capacity of AIS were determined using a 1 cm i.d. × 2 cm column of resin equilibrated in 10 mM Tris, pH 8·2 containing 0·1% bovine serum albumin (BSA, peptide-free, Research Products, Inc.). A control column of uncoupled Sepharose 4B was prepared in a similar manner. [^{125}I]-Insulin (porcine, 0·68 μCi/ml, 7·2 ng/ml, ICN) was dissolved in the Tris–BSA buffer and applied to the small column. Effluent was collected in 1 ml fraction and the amount of radioactivity determined in a Searle Analytic 1190 gamma counter. When radioactivity of the fractions reached a plateau value, it was assumed that maximum insulin binding had been reached. The column was then washed with Tris–BSA buffer until no radioactivity was detected in the effluent. Adsorbed [^{125}I]-insulin was eluted from the column with 1 M acetic acid. [^{125}I]-Gastrin (70 μCi/ml, 10 ng/ml, ICN) was processed through the AIS column in a similar manner. No binding of [^{125}I]-gastrin was observed. Uncoupled Sepharose did not bind either peptide.

The *Manduca* and *Apis* insulin-like immunoreactive fractions from gel filtration chromatography were dissolved in the Tris–BSA buffer and passed through separate larger AIS columns (1 cm i.d. × 6 cm). The columns were washed with 30 ml Tris–BSA buffer, followed by 20 ml of Tris only buffer and finally with 30 ml 1 M acetic acid. One millilitre fractions were collected. A 0·2 ml aliquot was removed from each fraction, lyophilized and subjected to insulin radioimmunoassay. The remaining sample was pooled, lyophilized, rechromatographed on Bio-Gel P-30 (see above) and appropriate fractions were subjected to amino acid analysis if enough peptide was isolated.

Radioimmunoassay

Samples were subjected to insulin radioimmunoassay using standard kits (Radioassay Systems Laboratories, Carson, CA, ICN Medical Laboratories, Portland, OR, Amersham Corp., Arlington Heights, IL). Levels of peptide were obtained by evaluation of experimental data against standard curves prepared using porcine insulin. The limit of detection of porcine insulin was 200 pg using this procedure.

Amino acid analysis

Samples were hydrolyzed for 24 hr *in vacuo* in 6 M HCl containing 0·1% phenol. Hydrolysates were analyzed as their *o*-phthaldialdehyde derivatives (HILL *et al*., 1979) using a high performance liquid chromatography system consisting of a Varian 5020 pump, a Rheodyne 7120 injector valve, a Waters μBondapak C_{18} column (30 cm × 3·9 mm, i.d.), a Turner fluorometer, and a Hewlett-Packard 3385A printer–plotter automation system.

Biological assay

Hypotrehalosemic assays were performed using *M. sexta* larvae as described previously (KRAMER, 1980). Trehalose was purified by gel filtration and quantitated by the anthrone reaction (CARROLL *et al*., 1956) as modified by KRAMER *et al* (1978).

RESULTS

Purification of insulin-like peptide from M. sexta *haemolymph*

Manduca haemolymph was initially extracted with 3 M acetic acid at 4°C to render the peptide components suitable for radioimmunoassay and subsequent fractionation steps (KRAMER *et al*., 1977,

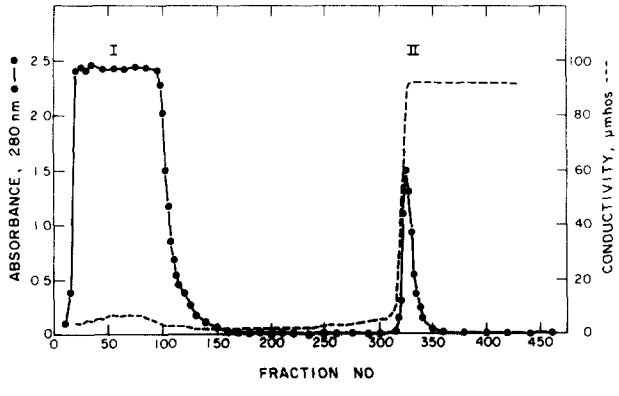

Fig. 1. Cation-exchange chromatography of acidic extract of *M. sexta* haemolymph on sulphopropyl Sephadex C-25. Lyophilized and reconstituted acidic extract was applied to a sulphopropyl Sephadex C-25 column (2 cm i.d. × 30 cm) equilibrated with 1·5 M acetic acid. Adsorbed material was eluted with the same buffer also containing 0·5 M NaCl. Absorbance at 280 nm —●—, conductivity, ---

1980). The acidic extract of haemolymph was next subjected to cation-exchange chromatography on sulphopropyl Sephadex C-25 at pH 3 (Fig. 1). Two sets of pooled fractions were collected; the unadsorbed material (I; fractions 20–100) and material that eluted with 0.5 M NaCl (II: fractions 320–345). Both fractions were dialyzed in low molecular weight cut off tubing against 0.1 M acetic acid, concentrated by lyophilization, redissolved in Tris–BSA buffer and aliquots of the pooled fraction were analyzed for immunoreactivity by using a standard porcine insulin assay (Fig. 2). Fraction II exhibited insulin immunoreactivity in a manner typical of standard mammalian insulin. The abilities of the serial-diluted fraction and porcine insulin to compete with [^{125}I]-insulin for binding to the antibody decreased in an identical manner Approximately 20 ng of insect insulin was recovered from 400 ml of haemolymph. No immunoreactivity was present in the unadsorbed fraction I.

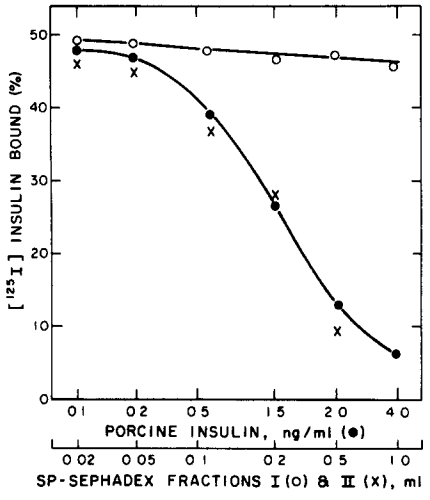

Fig.2. Radioimmunoassay of fractions I and II from cation-exchange chromatography of *M sexta* haemolymph. The immunoreactivity of porcine insulin (●) is compared to results with adsorbed (×) and unadsorbed (○) fractions from cation-exchange chromatography of haemolymph extract of *M. sexta*. The % [^{125}I]-insulin bound is plotted as a function of insulin concentration in standard (top) and volume of fraction (bottom) samples

Further purification of the insect insulin-like peptide was achieved by gel filtration on Bio-Gel P-10 in 0.1 M acetic acid (Fig. 3). When fraction II from the ion-exchange chromatography was processed, two peaks of apparent immunoreactivity eluted, one at fraction 10 and the other at fraction 16 The latter eluted in a position nearly identical to that of porcine insulin (fraction 15). The lower molecular weight immunoreactive component was pooled, lyophilized and further purified by affinity chromatography using a porcine insulin antibody as the binding species.

First, the binding capacity of the 6 ml AIS column was determined to be approximately 240 μg porcine insulin, exceeding the expected sample total by more than 1000-fold. The second immunoreactive insulin fraction from the Bio-Gel P-10 column was redissolved in Tris–BSA buffer pH 8.5 and passed over a virgin AIS column of equivalent size (Fig. 4). A significant quantity of material which absorbed in the u.v. region of the spectrum did not adsorb to the antibody column at pH 8.5 (fractions 5–27). When 1 M acetic acid was used as the mobile phase, a relatively small percentage of u.v.-absorbing material was released (fractions 49–55). The latter material was pooled, lyophilized and assayed for immunoreactive insulin. A total of 10 ng equivalents of immunoreactive insulin was obtained, corresponding to a yield of 0.5 ng/g of lyophilized haemolymph. A portion of that material (6 ng) was also subjected to acid hydrolysis and amino acid analysis after chromatography on a BioGel P-30 column (Fig. 5). Gel filtration was required to separate the immunoreactive peptide from some albumin (fractions 9–11) which also adsorbed to the affinity column. A single peak of immunoreactive insulin (5 ng, fractions 18–22) was eluted from the polyacrylamide gel column well separated from the bulk of the u.v.

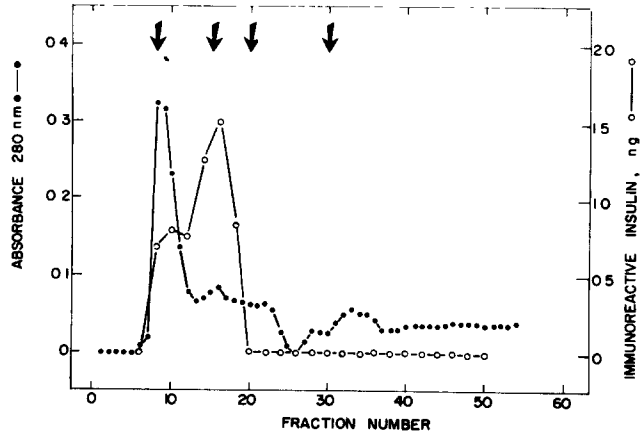

Fig. 3. Gel filtration of fraction II from cation-exchange chromatography of *M sexta* haemolymph extract on Bio-Gel P-10. Lyophilized and reconstituted acidic extract was applied to a Bio-Gel P-10 column (2.5 cm i.d × 45 cm) and eluted with 3 M acetic acid. The arrows indicate, from left to right, elution volumes, determined by using a second equivalent column, corresponding to bovine serum albumin, [^{125}I]-porcine insulin, glucagon and [^{14}C]-leucine. Absorbance at 280 nm, —●—, insulin immunoreactivity, —○—.

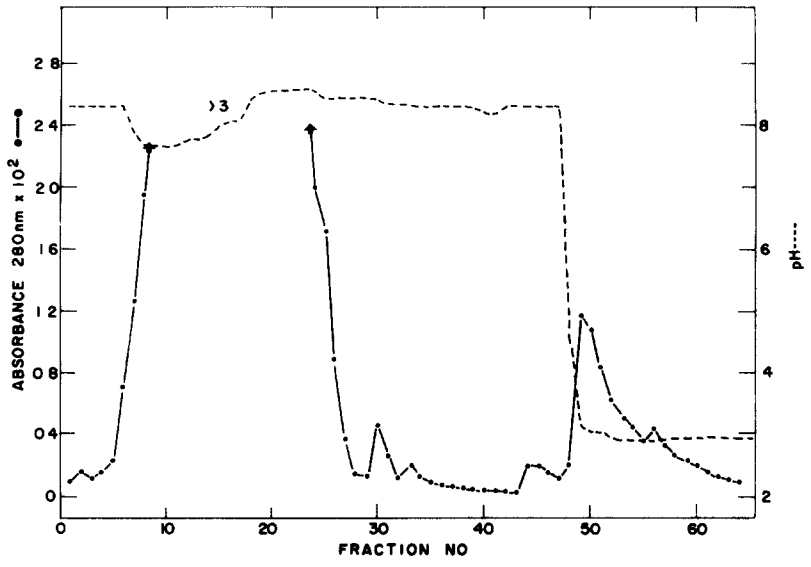

Fig 4 Immunoaffinity chromatography of fraction II from gel filtration of second fraction from cation-exchange chromatography of *M sexta* haemolymph extract on goat antiporcine insulin immunoglobulin-agarose Lyophilized and reconstituted fraction II from gel filtration was applied to the affinity column (1 cm i d ×6 cm) and eluted with (1) 10 mM Tris, 0 1% BSA, pH 8 2, (2) 10 mM Tris, pH 8 2 and (3) 1 M acetic acid Absorbance at 280 nm, —●—, pH, --- The aliquots of u v -absorbing material eluting around fraction 50 were lyophilized and subjected to insulin radioimmunoassay

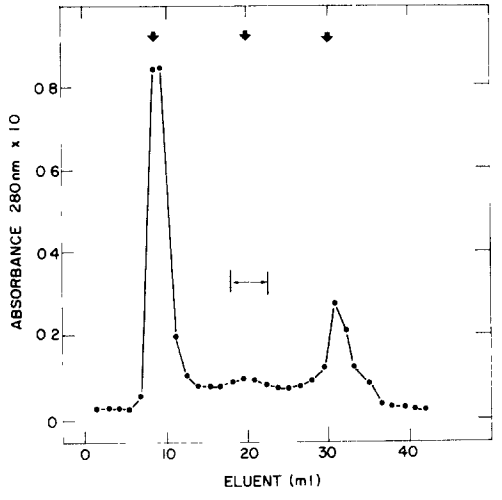

Fig 5 Gel filtration of immunoreactive fraction from immunoaffinity chromatography of *M sexta* haemolymph extract on Bio-Gel P-30 The lyophilized fraction was applied to a 0 9 cm i d ×70 cm column and eluted with 1 M acetic acid The arrows mark, from left to right, elution volumes, determined by using a second equivalent column, corresponding to bovine serum albumin, [^{125}I]-porcine insulin and [^{14}C]-leucine Absorbance at 280 nm, —●—

absorbing material No immunoreactivity was associated with albumin

Amino acid analysis of the *Manduca* haemolymph insulin-like peptide and comparison to that of porcine insulin revealed that the two materials were remarkably similar (Table 1) Most of the residues had the same molar ratios. The major differences occurred with serine, leucine and phenylalanine. All of the other residues were the same integer value or within one or two of each other. Also the ratio of basic to acidic amino acids was nearly identical. These results indicate that hornworm and porcine insulins are similar chemically

Biological assay

When the purified insulin-like peptide from *M. sexta* haemolymph was injected into fifth-instar larvae, hypotrehalosemia occurred (TAGER *et al.*, 1976). Injection of 2 ng immunoreactive peptide into each of two larvae reduced trehalose concentrations in haemolymph to 40 and 55% of the control level of 30 mM.

Separation of insulin-like peptide from A. mellifera *royal jelly*

In addition to extracting royal jelly with slightly alkaline and acidic pH buffers, it was also treated with diethyl ether to remove lipids that appeared to interfere with subsequent radioimmunoassay. The acidic extract was next subjected to chromatography on Bio-Gel P-30 in 1 M acetic acid (Fig. 6). To minimize the number of assays, samples were divided into seven pools of 15 tubes each (I–VII) and these were subjected to insulin radioimmunoassay. Immunoreactive insulin (IRI) was found in three of the eight pools. Amounts detected were 2.4 ng in pool II (void volume), 3.6 ng in VI (the same elution volume as porcine insulin) and 3.2 ng in VII (inclusion volume).

Each of the immunoreactive pools was fractionated by affinity chromatography. The immunoreactive insulin from royal jelly in pool II was adsorbed almost completely by the antibody column and was recovered in 70% yield. This immunoreactivity was composed of peptides with molecular weights equal to or greater than 3×10^4 and termed 'high molecular weight IRI' for the purpose of discussion. Not all of the immunoreactive

Table 2 Occurrence of insulin-like peptides in insects

Species	Source	Assay	Reference
Apis mellifera (honey-bee)	adult gut	RIA*	ISHAY *et al* (1976)
	larval midgut	RIA	ISHAY *et al.* (1976)
	larval midgut	RIA	MOREAU *et al.* (1981)
	whole body	RIA	MAIER *et al* (1978)
	royal jelly	Glucose oxidation in rat adipose tissue	DIXIT and PATEL (1964)
	worker jelly	Glucose oxidation in rat adipose tissue	DIXIT and PATEL (1964)
	royal jelly	RIA	KRAMER *et al* (1977)
	royal jelly	Hypotrehalosemia in *M sexta* larvae	KRAMER *et al.* (1977)
	royal jelly	RIA	This paper
Bombyx mori (silk moth)	brain, corpus cardiacum, corpus allatum	IC*	YUI *et al.* (1980)
Calliphora vomitoria (blowfly)	brain	RIA and hypotrehalosemia	DUVE and THORPE (1979)
		IC	DUVE *et al.* (1979)
Drosophila melanogaster (fruit-fly)	whole body	Hypoglycemia in mice	MENESES and ORTIZ (1975)
	haemolymph	RIA	SEECOF and DEWHURST (1974)
	head, whole body	RIA, glucose oxidation in rat adipocytes	LE ROITH *et al.* (1980, 1981)
Eristalis aeneus (hoverfly)	brain	IC	EL-SALHY *et al.* (1980)
Hymenopteran† *species*	gut	RIA	ISHAY *et al* (1976)
Locusta migratoria (locust)	corpus cardiacum	RIA, hypolipaemia	ORCHARD and LOUGHTON (1980)
Manduca sexta (tobacco hornworm)	haemolymph	RIA, amino acid analysis	KRAMER *et al.* (1980, this paper)
	corpus cardiacum/ corpus allatum complexes	RIA	TAGER *et al* (1976)
	corpus cardiacum/ corpus allatum complexes	Hypotrehalosemia in *M. sexta* larvae	TAGER *et al* (1976)
Periplaneta americana (cockroach)	corpus cardiacum/ corpus allatum complexes	RIA	TAGER *et al* (1976)
Plodia interpunctella (Indian meal moth)	head	RIA	TAGER *et al* (1976)

* RIA and IC are used as abbreviations for radioimmunoassay and immunocytochemistry, respectively

† A total of ten different hymenoteran species were examined including *Apis mellifera, Vespa orientalis, Vespa crabro, Paravespula vulgaris, Paravespula germanica, Dolichovespula media, Dolichovespula saxonica, Polyrachis simplex, Chalicodoma sicula,* and *Chrysis* sp

and DEWHURST, 1974) from *Drosophila melanogaster*; whole body (MAIER *et al.*, 1978), gut tissue (ISHAY *et al.*, 1976), royal jelly (DIXIT and PATEL, 1964; KRAMER *et al.*, 1977; this paper) and worker jelly (DIXIT and PATEL, 1964) from *Apis mellifera*; gut tissue from several hymenopteran species (ISHAY *et al.*, 1976), haemolymph and brain-corpus cardiacum–corpus allatum complex from *Manduca sexta* (TAGER *et al.*, 1976; KRAMER *et al.*, 1980); head from *Plodia interpunctella* (TAGER *et al.*, 1976); corpus cardiacum–corpus allatum from *Periplaneta americana* (TAGER *et al.*, 1976); corpus cardiacum from *Locusta migratoria* (ORCHARD and LOUGHTON, 1980); and brain from *Calliphora vomitoria* (DUVE *et al.*, 1979). Levels detected range from picograms to more than micrograms per gram of tissue or millilitres of haemolymph. The peptide has been localized immunohistochemically to median neurosecretory cells of *C. vomitoria* (DUVE and THORPE, 1979), median neurosecretory cells and nerve fibres from them reaching the corpus cardiacum and corpus allatum of *Bombyx mori* (YUI *et al.*, 1980), and cells in the pars intercerebralis of *Eristalis aeneus* (EL-SALHY *et al.*, 1980) and *Manduca sexta* (M. EL-SALHY, personal communication).

The specific localization of insulin-like peptides in brain tissue and haemolymph of insects is somewhat reminiscent of the vertebrate case where insulin has not only been found in pancreas, blood, and gastrointestinal tract, but also in brain (HAVRANKOVA *et al.*, 1978; ENG and YALOW, 1980). The identification of insulin-like and other vertebrate hormone-like peptides in tissues of animals as diverse as insects and mammals further suggests that the peptides and their anatomical distribution arose early during the evolution of eukaryotes. Whether the presence of insulin in certain tissues such as brain is due to its production *in situ* or its uptake and sequestration from plasma remains open to question (ENG and YALOW 1980; ROSENZWEIG *et al.*, 1980).

Considering the extreme sensitivity of the insulin RIA and the low concentration of insulin-like peptides found in our samples, it was very important to take precautions to minimize the possibility of contamination of our fraction with traces of mammalian insulin. First, new and disposable equipment was utilized whenever possible. Second, processing of solvents and buffers without tissue through the purification procedure did not yield immunoreactive substances. Third, the results from separate laboratories reporting an insulin immunoreactive peptide in other species of insects (see Table 2) demonstrates that it or a similar factor may be ubiquitous to insects.

Table 1 Amino acid compositions of *Manduca* and porcine insulins

Amino acid	*Manduca*	Porcine*
Aspartic acid	3 1	3
Threonine + glycine	8 0†	6 0
Serine	6 8	3
Glutamic acid	6 2	7
Proline	n d †	(1)
Alanine	2 0‡	2
Valine	2 9	3
Methionine	n d †	0
Isoleucine	2 1	2
Leucine	1 8	6
Tyrosine	2 8	4
Phenylalanine	1 0	3
Histidine	2 1	2
Lysine	0 9	1
Arginine	1 1	1
Tryptophan	n d †	0
Cysteine	n d †	(6)
Total	40 8	43(50)
Ratio, basic acidic	0 44	0 40

* From DAYHOFF (1972)

† Threonine and glycine not resolved and proline not detected by HPLC analysis. Methionine, tryptophan and cysteine not stable to acid hydrolysis. For comparison to porcine peptide, all residues except the above were utilized

‡ Averaged values from two analyses Integer values were normalized to 2.0 residues of alanine

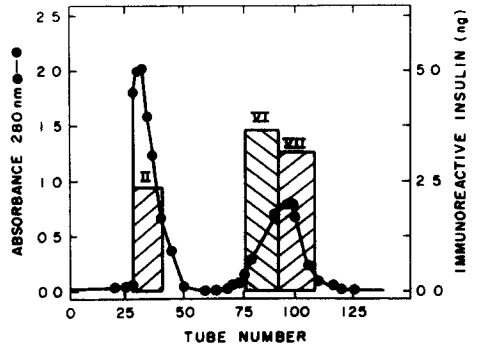

Fig 6. Gel filtration of acidic extract of *A mellifera* royal jelly on BioGel P-30. Lyophilized and reconstituted acid extract was applied to a Bio-Gel P-30 column (2.5 cm i.d. × 90 cm) and eluted with 3 M acetic acid. Absorbance at 280 nm, —●—, insulin immunoreactivity, cross-hatched area

insulin from the royal jelly pools VI and VII adsorbed to the affinity column. In fact, no adsorption was observed in pool VII. Pool VI contained both true immunoreactive insulin (3.3 ng) which was adsorbed by the column antibody and also some component(s) that interfered nonspecifically in the radioimmunoassay (0.3 ng) and was not fractionated by affinity chromatography The immunoreactive insulin had a molecular weight similar to or slightly less than vertebrate insulin. Pools VI and VII eluted just before and at the inclusion volume, respectively. The nonspecific immunoassay interfering material in pools VI and VII was probably low molecular weight substances which degraded the tracer or inhibited its binding in the batch RIA assay

Yields of adsorbed IRI from 200 g of freeze-dried royal jelly were 1 7, 3 3, and 0 ng for pools II, VI and VII, respectively, or a total of 25 pg/g Thus, the percentage of dry weight of adsorbed IRI in native royal jelly was about 10^{-9}% No amino acid composition or biological activity was determined for the royal jelly peptide due to the low amounts of material isolated. Previously, KRAMER *et al* (1977) demonstrated hypotrehalosemic activity in an aqueous extract of *A mellifera* royal jelly.

DISCUSSION

NORMANN (1975) suggested that hypertrehalosemia resulting from decapitation of the blowfly, *Calliphora erythrocephala*, was due to a lack of hypotrehalosemic hormone of cephalic origin The cephalic neuroendocrine gland complex, the corpus cardiacum–corpus allatum (CC–CA), was a likely release site for this hormone. Other investigators have also demonstrated hypertrehalosemia in other Diptera after selective removal of the corpus cardiacum of the blowfly, *Phormia regina* (CHEN and FRIEDMAN, 1977) or of the CC–CA in the housefly, *Musca domestica* (L.) (LUI, 1973). About the same time, an insulin-like peptide was detected in neuroendocrine tissues and haemolymph from other dipteran species (SEECOF and DEWHURST, 1974, DUVE *et al.*, 1979, DUVE and THORPE, 1979, EL-SALHY *et al.*, 1980). These results suggest that the dipteran hypotrehalosemic hormone and the insulin-like peptide are one and the same. Now similar evidence is available from lepidopteran species (Table 2, TAGER *et al.*, 1976, KRAMER *et al.*, 1980, this paper, YUI *et al.*, 1980; M. EL-SAHLY, personal communication). All of the above evidence points to an insulin functioning in insect carbohydrate metabolism. A recent report also indicates that a locust insulin-like peptide exhibits hypolipaemic activity as well (ORCHARD and LOUGHTON, 1980).

Insulin-like peptides have been identified previously by immunological, immunohistochemical, and biological assays in four orders of Insecta (Table 2). Hymenoptera (DIXIT and PATEL, 1964; ISHAY *et al.*, 1976, YUI *et al*, 1980, MOREAU *et al.*, 1981), Orthoptera (TAGER *et al*, 1976, ORCHARD and LOUGHTON, 1980); Diptera (SEECOF and DEWHURST, 1974, MENESES and ORTIZ, 1975, DUVE and THORPE, 1979, DUVE *et al*, 1979; LE ROTH *et al.*, 1980, EL-SALHY *et al.*, 1980) and Lepidoptera (TAGER *et al.*, 1976; YUI *et al.*, 1980; KRAMER *et al.*, 1980). The results of this investigation have confirmed the presence of immunoreactive insulin in *M. sexta* and *A. mellifera*, and report for the first time the amino acid composition of an insulin-like peptide isolated from an insect. The material called insect insulin is similar to mammalian insulin in its solubility in acid, elution profile on BioGel P-10 and P-30 (molecular size), cross-reactivity with anti-insulin immunoglobulin, biological activity, and amino acid composition.

Insulin-like petides have been semiquantitated in whole body (MENESES and ORTIZ, 1975), heads (LE ROITH *et al.*, 1980, 1981) and haemolymph (SEECOF

The presence of insulin-like material has been confirmed in other invertebrates (for review see FALKMER *et al.*, 1973, 1981) and also several unicellular eukaryotes (a ciliated protozoan and two unicellular fungi, LE ROITH *et al.*, 1980). These results suggest that a hormone can be highly conserved over extremely long periods of evolution (perhaps 900 million years) or that late recombination events can occur involving extramurally introduced genes

A peptide that had similar characteristics to bovine insulin was first separated from royal jelly using paper chromatography and paper electrophoresis by DIXIT and PATEL (1964). It produced an insulin-like effect in rat adipose tissue, increasing glucose utilization and oxidation. KRAMER *et al.* (1977) found that injection of aqueous extracts of royal jelly into *M sexta* larvae caused hypotrehalosemia ISHAY *et al.* (1976) and KRAMER *et al* (1977) experienced problems with radioimmunoassay of insect insulin. They could not detect immunoreactive insulin in native royal jelly Only after subjecting the jelly to acid extraction, delipidation and gel filtration were KRAMER *et al.* (1977) able to detect nanogram amounts of peptide. In the present study, the KRAMER *et al.* (1977) procedure was repeated, but only picogram levels of immunoreactive insulin were detected. The difference could be attributed to different sample sources, handling conditions, immunoassays, and/or the addition of an extra step in sample work-up, i.e. the affinity chromatographic separation. KRAMER *et al* (1977) obtained their royal jelly from U.S. suppliers and used a rabbit anti-bovine insulin radioimmunoassay system, while this study used royal jelly from a company in Taiwan and a guinea pig anti-porcine insulin system. It was also determined that estimates of insect insulin obtained from partially purified royal jelly samples had to be corrected for the presence of falsely positive material. The use of a fourth clean-up step, affinity chromatography, demonstrated that only 40% of apparent immunoreactive insulin detected after gel filtration was true immunoreactive insulin. The advantage of gel filtration followed by affinity chromatography was that it allowed discrimination of a true immunoreactive species according to molecular size. Royal jelly contained both an immunoreactive insulin similar in size to vertebrate insulin and a larger immunoreactive species, perhaps chemically similar to vertebrate proinsulin.

Preliminary experiments with untreated *M. sexta* haemolymph showed that some peptide clean-up procedure was necessary before a valid radioimmunoassay could be conducted. Based on previous experience with royal jelly IRI, it was decided to utilize affinity chromatography of a haemolymph acidic extract for such a clean-up. This was successful and haemolymph was found to contain approximately 0.5 ng IRI per g dry weight. This amounted to picomolar or greater concentrations in native haemolymph. Of the IRI 70% was peptide material with an apparent molecular weight of 5×10^3. The remaining IRI was of a large molecular weight and might be some form of prohormone.

This study using insect haemolymph and royal jelly describes the first attempt to isolate insulin from insect tissues using affinity chromatography. This procedure not only fractionated the low insulin content of our starting material, but also concentrated it to a level where radioimmunoassay and amino acid analysis could be carried out.

The quantities of insect IRI fractionated here were quite small because of the limited amount of tissue used If larger quantities of tissue become available, procedures used here can be scaled up and used with other conventional purification steps to yield enough purified IRI for further chemical characterization such as sequence analyses. If the amino acid sequence of insect IRI were found to compare favourably with that of vertebrate insulin, then this result would constitute the best evidence for the presence of insulin in insects. Also this material could be used in physiological studies to determine more precisely its biological activities.

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Note added in proof

LEROITH, SHILOACH, ROTH and LESNIAK (*J biol Chem.* **256,** 6533–6536, 1–1981) have recently demonstrated that insulin or a closely related molecule is native to the bacterium *Escherichia coli*